Bio-Plex Pro™ Human Apolipoprotein Assays 10-Plex Panel

Instruction Manual

For technical support, call your local Bio-Rad office,
or in the U.S., call 1-800-424-6723. For research use only.

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Table of Contents

Section 1
Introduction

Bio-Plex Pro™ Human Apolipoprotein Assays are sensitive magnetic bead–based multiplex assays that allow the accurate measurement of a range of biologically relevant apolipoproteins involved in cardiovascular and neurological diseases in diverse matrices, including serum, plasma, and culture supernatant. The multiplexing feature makes it possible to quantitate the levels of multiple analytes in a single well of a 96-well microplate in just 3 hours, using as little as 10 µl of serum or plasma.

These assays are offered in a convenient kit format that includes all necessary reagents, buffers, standards, and controls in single box.

The Bio-Plex Pro Human Apolipoprotein Assays include a wide range of proteins implicated in the pathophysiology of cardiovascular diseases, diabetes, and Alzheimer's disease. For researchers working with limited sample volume, the capacity to multiplex provides an effective option over the traditional ELISA method. The use of magnetic (MagPlex) beads allows researchers to automate wash steps on a Bio-Plex Pro (or similar) wash station.

Visit our website at **bio-rad.com/web/bio-plex** for a current listing of Bio-Plex Pro Assays.

Section 2
Principle

Technology

The Bio-Plex® Multiplex Immunoassay System is built around three core technologies. The first is a novel technology that uses up to 100 unique fluorescently dyed beads (xMAP technology) that permit the simultaneous detection of as many as 100 different types of molecules in a single well of a 96-well microplate. The second is a flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. The third is a high-speed digital signal processor that efficiently manages the fluorescent output.

Assay Format

The principle of these bead-based assays formatted for 96-well plates is similar to that of a capture sandwich immunoassay. An antibody directed against the desired target is covalently coupled to internally dyed beads.

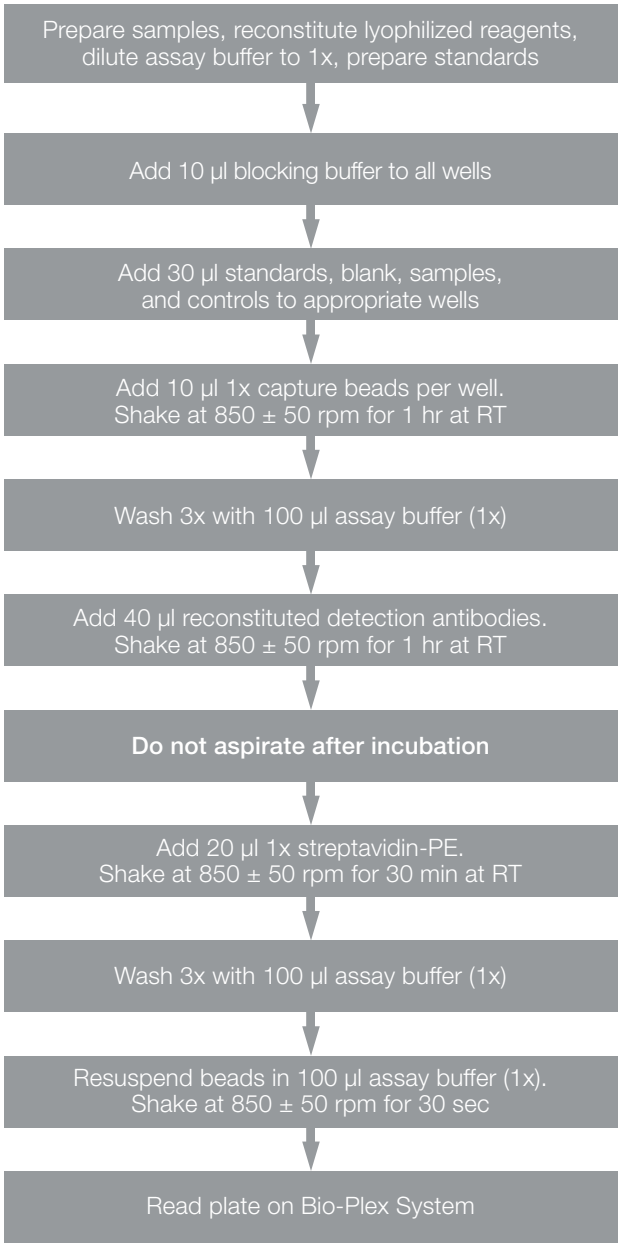
The coupled beads are allowed to react with a sample containing the target apolipoprotein. After a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope is added to the reaction. The result is the formation of a sandwich of antibodies around the target apolipoprotein. Streptavidin-phycoerythrin (SA-PE) is then added to bind to the biotinylated detection antibodies on the bead surface.

Data Acquisition and Analysis

Data from the reaction are then acquired using the Bio-Plex Multiplex Immunoassay System (or Luminex System), a dual-laser, flow-based microplate reader system. The contents of the well are drawn up into the reader.

The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent signal on the bead surface. This identifies each assay and provides data for a report on the level of target protein in the well. Intensity of fluorescence detected on the beads indicates the relative quantity of targeted molecules. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity on Bio-Plex Manager™ Software, the accompanying software package.

Assay Workflow



Section 3
Required Materials

Bio-Plex Pro™ Human Apolipoprotein Assays are offered in a convenient kit format that includes assay, reagent, control, and diluent components all in a single box. Analyzing these assays requires the use of Bio-Plex Manager™ Software.

Kit contents.

Component	Units	Volume	Description
Coupled magnetic beads (1x)	1	1.4 ml	2 ml, black vial
Detection antibodies	1	Lyophilized	5 ml glass, red crimp
Standard	1	Lyophilized	2 ml glass, silver crimp
Control 1	1	Lyophilized	2 ml glass, red crimp
Control 2	1	Lyophilized	2 ml glass, blue crimp
Standard diluent	1	Lyophilized	5 ml glass, green crimp
Sample dilution buffer	1	100 ml	125 ml bottle
Assay buffer (10x)	1	60 ml	60 ml bottle
Blocking Buffer	1	Lyophilized	5 ml glass, silver crimp
Streptavidin-PE (10x)	1	0.25 ml	0.5 ml, amber vial
Flat bottom plate (96-well)	1		
Plate seals	1		1 pack of 4

Storage and Stability

Kit components should be stored at 2–8ºC and should never be frozen. Coupled magnetic beads and SA-PE should be stored in the dark. All components are guaranteed for up to 6 months from the date of purchase when stored as specified here and on the package label.

Section 4
Recommended Materials

For optimal results, the use of the items below is recommended.

Recommended materials.

Item	Ordering Information
Bio-Plex® Multiplex Immunoassay System (or Luminex System)	Bio-Rad catalog #171000205
Bio-Plex Validation Kit	Bio-Rad catalog #171203001
Bio-Plex Calibration Kit	Bio-Rad catalog #171203060
Microtiter Plate Shaker	
IKA-Schuttler MTS-4 Shaker for 4 microplates or Lab-Line Model 4625 Plate Shaker (or equivalent, capable of 300–1,100 rpm)	IKA catalog #3208000 VWR catalog #57019-600
Vortexer	
VWR brand mini-vortexer	VWR catalog #58816-121
Scientific Instruments Vortex-Genie 2 Mixer	VWR catalog #58815-234
Reagent Reservoirs Box of 40 packages, 5 reservoirs per pkg (200 reservoirs), 50 ml capacity, graduated, polystyrene	Bio-Rad catalog #2244872
Tubes and Plates	
Titertube® Micro Test Tubes, pkg of 10 racks, 96 micro test tubes per rack (960 tubes), 1 ml, 8.8 x 45 mm, presterilized, not autoclavable	Bio-Rad catalog #2239395
Micro Test Tubes, pkg of 500, standard micro test tube, capped, 1.5 ml, autoclavable, natural	Bio-Rad catalog #2239501
Bio-Plex Pro™ Flat Bottom Plates, pkg of 40, 96-well plates, for use with Bio-Plex Pro wash stations when using magnetic bead–based assays	Bio-Rad catalog #171025001
Other Materials	
Pipets and pipet tips, 15 ml culture tubes, sterile distilled water, aluminum foil, absorbent paper towels	

Section 5
Sample Preparation

This section provides instructions for preparing samples derived from serum, plasma, and culture supernatant. For sample preparations not mentioned here, go to **bio-rad.com** to consult the publications listed in Bio-Rad bulletin 5297, which is also available for download.

Serum and Plasma Samples

Note that for plasma samples, EDTA tubes are recommended, although sodium citrate tubes are acceptable. Extremely lipemic samples may be filtered with a 0.22 µm filter to prevent clogging. Hemolyzed samples are not suitable for Bio-Plex Pro™ Human Apolipoprotein Assays.

1. Collect and process the serum or plasma samples and assay immediately or freeze at –20ºC. Avoid repeat freezing and thawing.
2. Centrifuge the samples at 1,000 rpm for 15 min at 4ºC to clear the samples of precipitate. Alternatively, carefully filter the samples with a 0.22 µm filter to prevent instrument clogging.
3. Immediately dilute sample 1:50,000 with sample diluent (see table below). Keep the diluted samples on ice until ready for use.

Preparation of the sample dilutions.

Sample Dilution	Volume of Sample, µl		Volume of Sample Diluent, µl
1:50,000	(a) Prepare 1:10	10	90
	(b) Prepare 1:50	10 (a)	490
	Prepare 1:100	5 (b)	495

Note: Controls are ready to use after reconstitution. No dilution is needed.

Section 6
Standard Preparation

One vial of lyophilized apolipoprotein standard is provided in each Bio-Plex Pro™ Human Apolipoprotein Assay Kit. The product data sheet provided with each assay lists the concentration of the reconstituted standard. This procedure will prepare enough standard to run each dilution in duplicate.

Reagent Preparation

Reconstitution of the lyophilized reagents.

Reagent	Volume dH_2O
Standards mix	150 µl
Control 1	100 µl
Control 2	100 µl
Blocking buffer	1.5 ml
Standard diluent	1.0 ml
Detection antibodies	4.8 ml

1. Allow vial of apolipoprotein standard to sit at room temperature for 5 min.
2. After adding dH_2O to the reagents as described in Table 1, mix by vortexing at medium speed.
3. Bring the 10x assay buffer to ambient temperature (RT).
4. Mix by inversion to ensure all salts are in solution.
5. Prepare 1x assay buffer by diluting one part 10x assay buffer (60 ml) with nine parts dH_2O (540 ml).

Reconstitute Standard

1. Gently tap the glass vial containing the lyophilized apolipoprotein standard on a solid surface to ensure the pellet is at the bottom.
2. Reconstitute 1 vial of lyophilized standard with 150 µl of dH_2O.
3. Allow vial to sit at room temperature for 5 min.
4. Gently vortex for 1–3 sec and incubate on ice for 30 min. Be consistent with the incubation time for optimal assay performance.

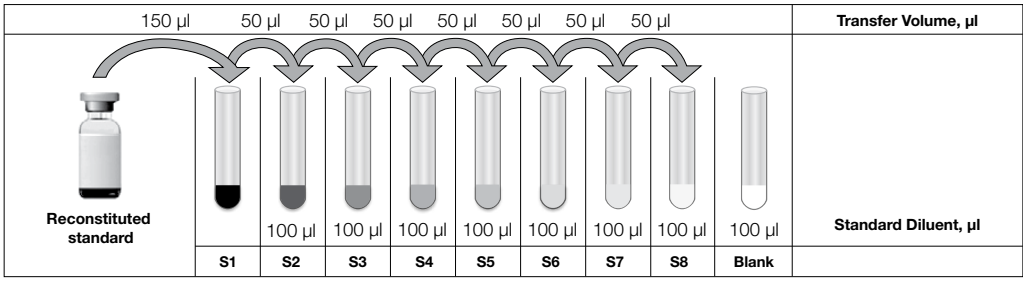
Prepare Standard Dilution Series

The analyte concentrations specified for the eight-point standard dilution set have been selected for optimized curve fitting using the five-parameter logistic (5PL) or four-parameter logistic (4PL) regression in Bio-Plex Manager™ Software. Results generated using dilution points other than those listed in this manual have not been optimized.

1. Label a set of 1.5 ml Eppendorf tubes as shown in the diagram below.
2. Pipet the appropriate volume of standard diluent into the tubes (see diagram below).
3. Transfer the reconstituted standards vial to the first 1.5 ml tube labeled “S1.” Vortex gently. This is identified as S1 in the diagram below and in the product insert provided with the assays.
4. Continue making serial dilutions of the standard as shown. After making each dilution, vortex gently. Change the pipet tip after every transfer.

   Note: Running an additional two 0 pg/ml blanks is strongly recommended. Use 50 µl of the standard diluent as the blank sample. The 0 pg/ml points should be formatted as blanks, not as points on the curve when using Bio-Plex Manager Software. The blank wells are also useful for troubleshooting and determining the limit of detection (LOD).
5. Keep the standards on ice until ready for use. Standards should be used immediately and should not be frozen for future use.

Standard dilution series.



* Refer to the product data sheet for the S1 value for each analyte. Each standard is a fourfold dilution of the preceding one.

Section 7
Control Preparation (Optional)

Two vials of lyophilized controls are provided in each Bio-Plex Pro™ Human Apolipoprotein Assay Kit. Controls are provided in high and low concentrations to monitor plate-to-plate variations. This section contains instructions on how to reconstitute the lyophilized controls. The product insert provided with the assays lists the concentration ranges of the reconstituted controls. The reconstituted controls can then be further diluted to prepare any concentration of user-specified quality controls. To ensure optimal assay performance, the controls should be prepared in a manner consistent with that used to prepare the apolipoprotein standards.

Reconstitute Controls

1. Gently tap the glass vial containing one of the lyophilized controls on a solid surface to ensure the pellet is at the bottom.
2. Reconstitute vial with 100 µl of dH_2O. Do not use assay buffer to dilute controls.
3. Allow vials to incubate at room temperature for 5 min. Be consistent with the incubation time to ensure optimal assay performance.
4. Refer to the product data sheet provided with the assay for the concentration range of each reconstituted control.

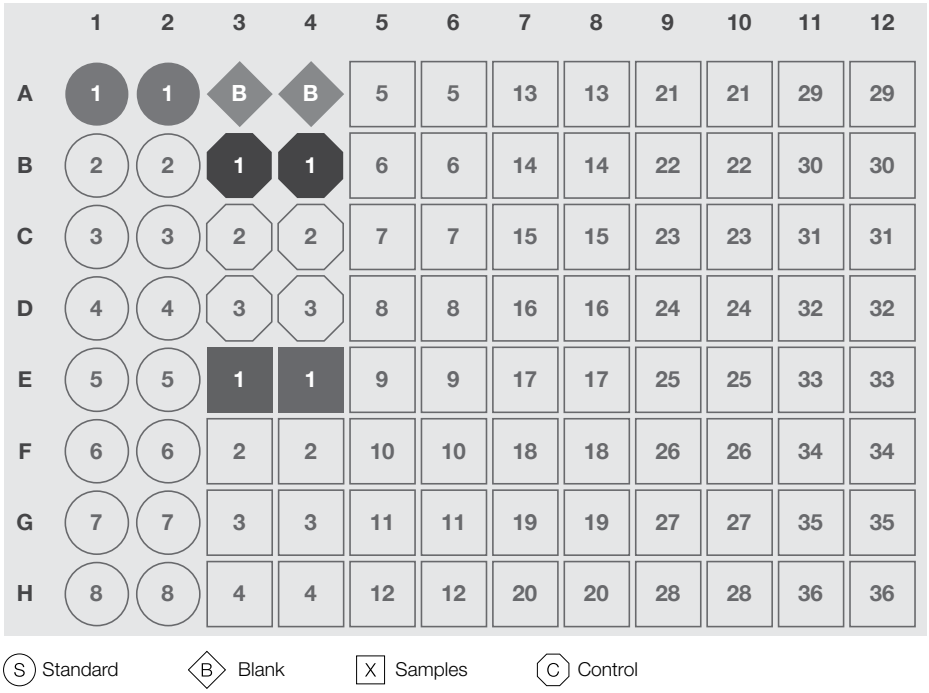
Section 8
Assay Instructions

The following instructions apply to Bio-Plex Pro™ Human Apolipoprotein Assays. All of the necessary components are provided premixed for ease of use.

Plan Experiment

1. Assign wells of a 96-well plate for each standard, control, and sample (see the example below).
2. Determine the total number of wells to be used in the assay. Include 25% excess (or two additional wells for every eight wells used) to ensure that enough coupled beads, detection antibodies, and SA-PE are prepared.

Example plate.



Assay Procedure

Bring all buffers to room temperature. Avoid bubbles when pipetting.

Assay Key. The following terms are repeated throughout the assay procedure. Refer to these detailed instructions when **wash** and **incubate** are shown in bold.

Term	Detailed Directions
Wash	Add 100 µl of assay buffer to each well. Wash using Bio-Plex Pro Wash Station automated wash station or by using a handheld magnetic plate washer. When using the handheld magnetic washer, ensure plate is securely attached; invert plate and shake out volume from wells to wash.
Incubate	Gently cover the plate with a new sheet of sealing tape. Place the plate on a microplate shaker and then cover with aluminum foil. Shake the plate at room temperature at 850 ± 50 rpm for the specified incubation time.

1. Equilibrate the diluted standards, samples, and controls at room temperature for 20 min prior to use.
2. Add 10 µl of blocking buffer to all wells of the plate.
3. Add 30 µl of the standard, controls, sample, or blank to the appropriate wells of the plate.
4. Vortex the coupled magnetic beads for 10–20 sec at medium speed. Add 10 µl to each well. A multichannel pipet is recommended. If using a single-channel pipet, vortex frequently to keep beads in solution.
5. Cover the plate with a plate seal and protect it from light with aluminum foil. Incubate on a shaker at 850 ± 50 rpm for 1 hr at RT.
6. Wash the plate three times with 100 µl per well of the 1x assay buffer.
7. Vortex the reconstituted detection antibodies at medium speed for 10–20 sec. Add 40 µl to each well.
8. Cover plate with plate seal and protect from light with aluminum foil. Incubate on shaker at 850 ± 50 rpm for 1 hr at room temperature. Do not aspirate after incubation.
9. Prepare 1:10 dilution of SA-PE. Add 225 µl of 10x SA-PE to 2,025 µl of 1x assay buffer for a total volume of 2,250 µl of 1:10 SA-PE solution.
10. Add 20 µl of the diluted SA-PE to each well of the plate.
11. Cover plate with plate seal and protect from light with aluminum foil. Incubate on shaker at 850 ± 50 rpm for 30 min at room temperature.
12. Wash three times with 100 µl of 1x assay buffer.
13. Add 100 µl of 1x assay buffer to each well. Incubate for 30 sec to resuspend the beads. Acquire the data immediately as described in Section 9.

Section 9
Data Acquisition

Bio-Plex Pro™ Human Apolipoprotein Assays require the use of Bio-Plex Manager™ Software version 4.1 or higher. Recommendations for acquiring data using the Bio-Plex® Suspension Array System are listed below. Alternatively, refer to the Bio-Plex Manager Software User Guide or instructions provided with the Luminex instrument.

Prepare System

1. Empty the waste bottle and fill the sheath fluid bottle before starting (if HTF not present). This will prevent fluidic system backup and potential data loss.
2. Turn on the reader and microplate platform (and HTF if present). Allow the system to warm up for 30 min.
3. Select **Start up** and follow the instructions to prepare the reader to acquire data. If the system is idle for 4 hr, the lasers will automatically turn off and a 30 min warm-up period will again be required prior to acquiring data. Select **Warm up** and wait for the optics to reach operational temperature.

Calibrate

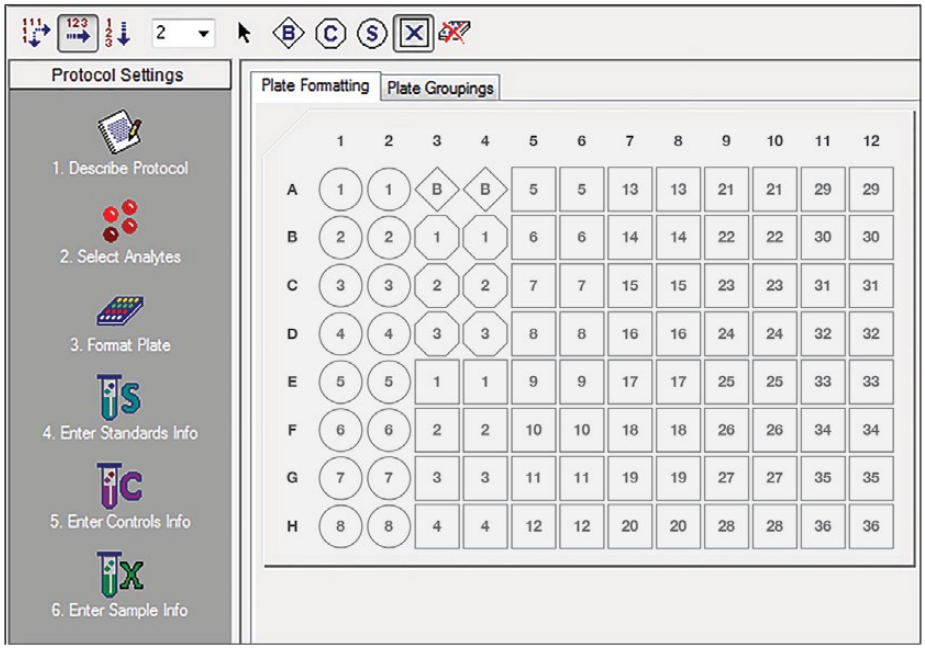
Calibrate using Bio-Plex Calibration Beads and target values. Daily calibration is recommended before acquiring data.

1. Select **Calibrate** and confirm that the default values for CAL1 and CAL2 are the same as the values on the Bio-Plex Calibration Bead labels. Use the Bio-Plex low RP1 target value for CAL2 calibration for Bio-Plex Pro Human Apolipoprotein Assays.
2. Select **OK** and follow the instructions for CAL1 and CAL2 calibration.

Prepare Protocol

1. Open a new protocol by selecting **File > New** from the main menu. Locate the steps at the left of the protocol menu.

   Note: To minimize data entry, go to **bio-rad.com/web/bio-plex** to download preset lot-specific Bio-Plex Pro Human Apolipoprotein Assay protocols.
2. Select **Step 1 (Describe Protocol)** and enter information about the assay.
3. Select **Step 2 (Select Analytes)** and select the panel for Apolipoproteins. Choose the target proteins for the assays on the plate. Note that this information will already be entered with the preset downloaded protocol.



4. Select **Step 3 (Format Plate)** and click the Plate Formatting tab. Click (S) and drag the cursor over all wells that contain standards. Then click (B) and drag the cursor over wells that contain blanks. Repeat with (C) to identify all wells that contain controls and [X] to identify all wells that contain samples.

   Note: If the preset protocol was downloaded, a formatted plate will already be provided. Make any necessary changes to the preset formatted plate so that it matches your plate setup.

5. Select **Step 4 (Enter Standards Info)** to enter standards information. Note that this information will already be entered with the preset download protocol.
   a. Select each analyte individually from the dropdown list.
   b. Select **Enter Automatically** and then select S1 as the most concentrated value.
   c. Enter the concentration of S1 from the product insert provided with the assay.
   d. Enter the dilution factor as 3 and select **Calculate**. The standard information for the selected analyte will be populated.
   e. Deselect the box for **Apply same concentration values for all analytes**. Repeat steps 5a–5d for each analyte in the assay.

6. Select **Step 5 (Enter Controls Info)** to enter controls information. This is where the concentration of the user-specified controls is entered in the protocol.
   a. Select each analyte individually from the dropdown list.
   b. Enter the description, concentration, and dilution information for each user-specified control.
   c. Deselect the box for **Apply same concentration values for all analytes**. Repeat steps 6a and 6b for each analyte in the assay.

7. Select **Step 6 (Enter Sample Info)** and enter sample information.

Acquire Data

1. Shake the assay plate at 850 ± 50 rpm for 30 sec immediately before acquiring data. Failure to do so will increase data acquisition time due to bead settling.
2. Visually inspect the plate and ensure that the assay wells are filled with buffer prior to placing the plate in the Bio-Plex Microplate platform.
3. Slowly remove the sealing tape and any plate cover before placing the plate in the reader.
4. Select **Step 7 (Run Protocol)**:
   a. Specify data acquisition for 100 beads per region.
   b. In Advanced Settings, set the Bead Map to 25 region.
      Note: Bio-Plex Pro Human Apolipoprotein Assays contain magnetic beads and require the use of the 25 region map available in Bio-Plex Manager Software version 4.1 or higher.
   c. In Advanced Settings, set the sample size to 50 µl.
   d. In Advanced Settings, confirm that the default DD gate values are set to 5,000 (low) and 25,000 (high).
      Note: When using a Luminex instrument, set the gates according to the procedure in the Luminex manual.
   e. Select **Start** and save the .rbx file. Then follow the instructions for data acquisition.
5. If acquiring data from more than one plate, empty the waste bottle and refill the sheath bottle after each plate (if HTF not present). Select **Wash Between Plates**  and follow the instructions for fluidics maintenance. Then repeat the Prepare Protocol and Acquire Data steps.

   Note: Use the Wash Between Plates command after every plate run to reduce the possibility of the instrument clogging.
6. When data acquisition is complete, select **Shut down**  and follow the instructions.

Reacquire Data

It is possible to acquire data from a well or plate a second time using the Rerun/ Recovery mode located below Start in Step 7 (Run Protocol).

1. Check the wells where data will be acquired a second time. Any previous data will be overwritten.
2. Aspirate assay buffer with the wash method of choice but do not perform the wash step.
3. Add 100 µl of assay buffer to each well. Cover the plate with a new sheet of sealing tape.
4. Repeat steps 1–6 from Acquire Data to acquire data a second time. The newly acquired data should be similar to the initially acquired data. However, the data acquisition time will be extended since fewer beads are present in each well.

Section 10
Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with Bio-Plex Pro™ Human Apolipoprotein Assays. If you experience any of the problems listed below, review the possible causes and solutions provided. This will assist you in resolving problems directly related to how the assay steps should be performed. Poor assay performance may also be due to problems with the Bio-Plex® Array Reader. To eliminate this possibility, we highly recommend use of the Bio-Plex Validation Kit. This kit will validate all the key functions of the array reader and assist the user in determining whether the array reader is functioning properly.

Possible Causes	Possible Solutions
High Inter-Assay CV	
Standards were not reconstituted consistently	Always be consistent with the incubation time and temperature.
Reconstituted standards and diluted samples were not stored properly	Prepare reconstituted standards and diluted samples on ice as instructed. Equilibrate the reconstituted standards and diluted samples to room temperature prior to plating.
Pipetting technique	Pipet carefully and slowly when adding standards, samples, detection antibodies, and SA-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.
Reagents and assay components were not equilibrated to room temperature prior to plating	Equilibrate all reagents and assay components to room temperature prior to plating
Contamination with wash buffer during wash steps	Be careful not to splash wash buffer from one well to another. Be sure that the wells are filtered completely and that no residual volume remains. Also, be sure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing.
Slow pipetting of samples and reagents across the plate	Sample pipetting across the entire plate should take less than 4 min. Reagent pipetting across the entire plate should take less than 1 min.
Low Bead Count	
Beads clumped in multiplex bead stock tube	Vortex for 15–20 sec at medium speed before aliquoting beads.
Did not shake plate enough before incubation steps and prior to reading	Shake the plate at 850 ± 50 rpm for 30 sec before incubation steps and immediately before reading the plate.
Reader is clogged	Refer to the troubleshooting guide in the Bio-Plex hardware instruction manual.
Low Signal or Poor Sensitivity	
Standards reconstituted incorrectly	Follow the apolipoprotein standard instructions carefully.
Detection antibody or SA-PE diluted incorrectly	Check your calculations and be careful to add the correct volumes.

Possible Causes	Possible Solutions
High Background Signal	
Incorrect buffer was used (for example, assay buffer used to dilute standards)	Use sample matrix or serum standard diluent to dilute apolipoprotein standards.
Spiked “0 pg/ml” wells by mistake	Be careful when spiking standards. Do not add any antigens in the 0 (blank) point.
SA-PE incubated too long	Follow the procedure incubation time.
Poor Recovery	
Expired Bio-Plex Reagents were used	Check that reagents have not expired. Use new or unexpired components.
Incorrect amounts of components were added	Check your calculations and be careful to add the correct volumes.
Microplate shaker set to an incorrect speed	Check the microplate shaker speed and use the recommended setting. Setting the speed too high may cause splashing and contamination. Use the recommended plate shaker.
Pipetting technique	Pipet carefully and slowly when adding standards, samples, detection antibodies, and SA-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

Section 11
Safety Considerations

Eye protection and gloves are recommended while using this product. Consult the SDS for additional information.

Human Source Material — Treat as Potentially Infectious

The Bio-Plex Pro™ Human Apolipoprotein Assays contain components of human origin. This material should be handled as if capable of transmitting infectious agents. Please use universal precautions. The material has been tested by an FDA-approved test and found negative for HBsAg, HIV 1/2 Ab, HIV-1 Ag, and HCV. No test method can provide total assurance that hepatitis B virus, hepatitis C virus, human immunodeficiency virus, or other infectious agents are absent. These components should be handled at Biosafety Level 2 containment (CDC (1999). Biosafety in Microbiological and Biomedical Laboratories). Handle Bio-Plex Pro serum and plasma standard diluents as potentially biohazardous material under at least Biosafety Level 2 containment.

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